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ORIGINAL PAPER

Lysophosphatidic acid-induced interleukin-1β expression is mediated through G_i/Rho and the generation of reactive oxygen species in macrophages

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Abstract Lysophophatidic acid (LPA), a low-molecular-weight lysophospholipid enriched in platelets and mildly oxidized low-density lipoproteins, is known to regulate inflammation and atherosclerosis by binding to its cognate receptors. In this study, we reported that LPA upregulated interleukin-1β (IL-1β) expression in mouse J774A.1 macrophages. By using pharmacological inhibitors, it was suggested that G_i/Rho activation and subsequent reactive oxygen species (ROS) production were involved in IL-1β induction. In addition, IL-1β induction by LPA was also observed in human primary macrophages. In summary, LPA is involved in the processes of inflammation by affecting macrophage behavior.

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Introduction

Lysophosphatidic acid (LPA) is a simple lysophospholipid with a variety of biological activities. Sources of LPA are mainly platelets and mildly oxidized low-density lipoproteins (mox-LDLs) [8, 30]. The biological effects elicited by LPA are mediated through recognition of LPA by a series of G protein-coupled LPA receptors, LPA_1, LPA_2, and LPA_3, encoded by endothelial differentiating genes [1].

In human endothelial cells, LPA facilitates wound healing; cell proliferation; cell migration; ICAM-1, interleukin-8 (IL-8) and MCP-1 expressions; matrix metalloprotease-2 (MMP-2) activation; and cell invasion [18, 20, 21, 32]. Recent studies demonstrated that LPA is an important regulator of atherosclerosis by activating human monocytic cells [4, 10] and inducing neointimal formation in a rat carotid artery model [34]. In addition, macrophages are also involved in the process of inflammation and atherosclerosis [22]. However, the relationship between LPA and macrophages is not clearly understood.

The present study demonstrated that in J774A.1 macrophages, LPA upregulated IL-1β expression by activating Rho- and G_i-dependent pathways. In addition, LPA-induced ROS production, which is downstream of G_i/Rho signals, was also involved in the production of IL-1β. Moreover, IL-1β was also elevated by LPA in human macrophages derived from umbilical cord blood. These results suggest that LPA regulates inflammation-related functions in both mouse and human macrophages.

Materials and methods

Reagents

1-Oleoyl-lysophosphatidic acid (LPA), pertussis toxin (PTx), actinomycin D, and N-acetylcysteine (NAC) were purchased from Sigma (St. Louis, MO). Diphenyleneiodonium chloride (DPI) was purchased from Tocris Cookson (Ellisville, MO). The C3 exozyme (C3) was purchased from BIOMOL (Plymouth Meeting, PA). The monoclonal rat anti-mouse IL-1β antibody was obtained from R&D System (Minneapolis, MN). TRIzol, the Superscript II kit, and dihydrorhodamine 123 (DHR-123) were purchased from Invitrogen (Grand Island, NY).

Cell line and culture

The murine macrophage cell line, J774A.1, was obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone, Logan, UT) at 37°C under a humidified atmosphere of 5% CO_2.

Umbilical Cord Blood (UCB) sample collection, Mononuclear Cell (MNC) processing, and macrophage generation

Term UCB was harvested with a standard 250-ml blood bag (Terumo, Shibuya-ku, Tokyo, Japan) with informed consent and was processed within 24 h. Buffy-coat cells were obtained, and then these cells were layered onto a Ficoll–Paque solution ($\rho = 1.077$ g/ml, Amersham Biosciences, Uppsala, Sweden) at $700\times g$ for 40 min. For macrophage generation, MNCs were incubated with macrophage induction medium (1:1 mixture of RPMI 1640 and Panserin 401 medium) supplemented with a cocktail of cytokines (25 ng/ml of GM-CSF and M-CSF) and 10% FBS. Cells were subcultured for experiments 1 week after induction. The purity of the macrophages was confirmed by flow cytometry.

LPA treatment

LPA, at 1 mM, was prepared in methanol and stored at −20°C. Cells were cultured at 75% confluence in complete medium, and then starved in serum-free medium overnight. LPA was added to serum-free RPMI 1640 containing 0.005% fatty acid-free bovine serum albumin (BSA) as a carrier.

RNA Isolation, Reverse-transcription Polymerase Chain Reaction (RT-PCR), and Real-time PCR analysis

Total cellular RNA was extracted from cells using the TRIzol reagent. Complementary DNAs were synthesized with an oligo-dT primer using a Superscript II kit. PCR products were resolved on 1.5% agarose gels stained with ethidium bromide and then photographed. Real-time PCR was carried out using an iCycler iQ real-time detection system (Bio-Rad, Hercules, CA) with iQ SYBR Green Supermix (Bio-Rad). The specificity was confirmed by melting-curve detection following the real-time PCR reaction. All gene-specific oligonucleotides sequences, annealing temperatures (Tm), and cycle numbers are described in Table 1.

Table 1 Primer sets

Primer	Sequences (F, forward; R, reverse)	Tm (°C)	Cycle	Size (bp)
Human	F: GGTGGTCTCCTCTGACTTCAAC	60	40	215
GAPDH*	R: TCTCTCTTCCTCTTGTGTTCTTG			
Human	F: CCGACCACCACTACAGCAAGG	60	40	88
IL-1β*	R: GGGCAGGGAACCAGCATCTTC			
Mouse	F: ACCACAGTCCATGCCATCAC	62	20	452
GAPDH	R: TCCACCACCCTGTTGCTGTA			
Mouse	F: TTGACGGACCCCAAAAGATG	62	30	204
IL-1β	R: AGAAGGTGCTCATGTCCTCA			
Mouse	F: AAGGCTGTGGGCAAGGTCATC	60	40	105
GAPDH*	R: CAGGCGGCACGTCAGATCC			
Mouse	F: GCCTCGTGCTGTCGGACC	60	40	114
IL-1β*	R: TGTCGTTGCTTGGTTCTCCTTG			

*Represents primers for real-time PCR

Western blot analysis

Cells were lysed on ice with RIPA buffer. Samples were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). The immunoreactive proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL) and quantified using TotalLab 2.01 software (Nonlinear, Durham, NC).

Intracellular Reactive Oxygen Species (ROS) measurement

Intracellular ROS were detected by flow cytometry using DHR-123 as a fluorescent probe for ROS. Starved cells were loaded with 10 μM DHR-123 in PBS for 30 min. Loaded cells were washed with PBS, followed by incubation with the indicated treatments for 30 min. The fluorescence intensities of different treatments were determined in 10,000 cells using a CyFlow flow cytometry (Partec, Muester, Germany). For quantification, the means of the histograms were calculated using the Flowmax program (Partec).

Enzyme-linked Immunosorbent Assay (ELISA) for human IL-1β

IL-1β of human macrophages was measured with an ELISA kit from Cayman (Ann Arbor, MI). In brief, 1×10^6 cells were seeded in a 6-cm dish with complete culture medium for 24 h. Cells were starved overnight, followed by treatment with 5 μM LPA or vehicle in a volume of 1.5 ml for 8 h. IL-1β levels of different treatments were assessed immediately after the supernatants were collected.

Statistical analysis

Each result was obtained from at least three independent experiments and is presented as the mean ± SD. Data were statistically analyzed using one-way analysis of variance (ANOVA), followed by Fisher's protected least-significant difference test.

Results

LPA-induced IL-1β expression in J774A.1 cells

Based on our preliminary studies, we demonstrated that LPA induced IL-1β expression in mouse peritoneal macrophages [19]. Thus, we further examined the detailed effects of LPA in mouse J774A.1 macrophages. By using the RT-PCR analysis, we observed that IL-1β mRNA levels were upregulated by LPA in concentration- and time-dependent manners (Fig. 1a, c, upper panel). The quantified results by real-time PCR analysis showed that IL-1β mRNA was significantly induced by 1 μM LPA, and the expression level peaked with 5 μM LPA treatment (Fig. 1a, lower panel). Furthermore, IL-1β mRNA expression was substantially induced by 2 h of LPA treatment (Fig. 1c, lower panel). At the protein level, LPA induced IL-1β protein expression in concentration- and time-dependent manners. Results from the dose-response experiment revealed that IL-1β protein was significantly induced by 1 μM LPA, and the induction peaked with 5 μM LPA treatment (Fig. 1b). On the other hand, time-course experiments showed that IL-1β protein was significantly enhanced at 4 h (Fig. 1d).

LPA-induced IL-1β Is mediated through G_i-, Rho-, and transcription-dependent Pathways

The effects of LPA are mainly mediated through G protein-coupled receptors, which activate downstream G proteins (reviewed in [2]). As shown in Fig. 2a, IL-1β induction by LPA was significantly attenuated in the presence of 1 μg/ml C3 or 15 ng/ml PTx [20], which are known to block Rho (downstream of G_{12})- and G_i-dependent signals. On the other hand, IL-1β could be regulated either at both transcriptional or post-translational levels [7]. By using actinomycin D [31], a general translation inhibitor, we demonstrated that LPA-induced IL-1β protein expression was completely suppressed (Fig. 2b). These results indicated that LPA regulates IL-1β expression is mostly at the transcriptional levels.

ROS are involved in LPA-enhanced IL-1β expression

ROS play important roles in inflammatory processes, and might function as secondary messengers in multiple signaling transduction pathways [9, 16]. In the presence of LPA, ROS were enhanced in J774A.1 cells (Fig. 3a). Additionally, ROS induction by LPA in mouse RAW264.7 macrophages and human primary macrophages was also observed (data not shown). On the other hand, C3 and PTx partially inhibited LPA-induced ROS production (Fig. 3b). To further assessed the relationship between IL-1β and LPA's enhancement of ROS, experiments with NAC (an antioxidant) and DPI (an inhibitor of ROS-generating enzyme, NADPH oxidase) were conducted. In the presence of 10mM NAC or 10 μM DPI [4, 14], LPA-enhanced ROS were entirely suppressed (Fig. 3c). Likewise, LPA-induced IL-1β was significantly inhibited when cells were pretreated

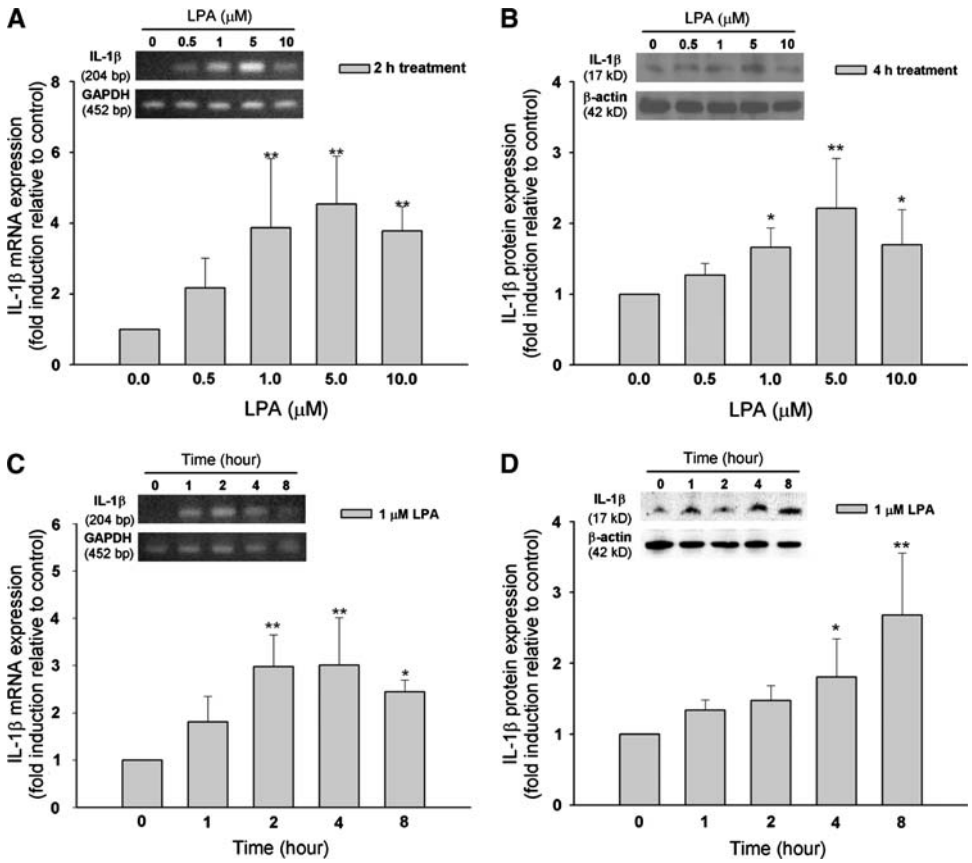

Fig. 1 Lysophosphatidic acid (LPA) enhances interleukin (IL)-1β expression in J774A.1 cells. To examine the effects of LPA on IL-1β expression, cells were treated as indicated. At the transcriptional level, mRNA was subjected to RT-PCR and real-time PCR analysis. At the translational level, total cell lysates were collected and subjected to Western blot analysis. (**a**, **b**) Dose response of LPA-induced IL-1β expression. (**c**, **d**) Time course of LPA-induced IL-1β expression. Images of agarose gels and immunoblotting films from one representative experiment are shown here. Each bar of the histogram represents quantified results from three independent experiments and is shown as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the control level

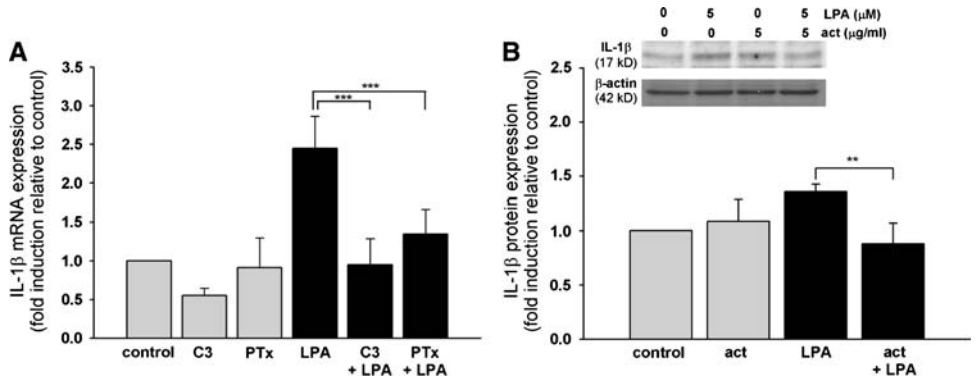

Fig. 2 Lysophosphatidic acid (LPA)-induced interleukin (IL)-1β(is mediated through Rho-, G_i-, and transcription-dependent pathways. (**a**) Starved J774A.1 cells were pretreated with 1 μg/ml C3 or 15 ng/ml pertussis toxin (PTx) overnight followed by 1 μM LPA treatment for 2 h. IL-1β expression levels were analyzed by real-time PCR. (**b**) Starved J774A.1 cells were pretreated with 5 μg/ml actinomycin D (act) for 1 h followed by incubation with 5 μM LPA for 4 h. IL-1β protein levels were analyzed by Western blot. $^{**}P < 0.01$, $^{***}P < 0.001$

with NAC or DPI, which indicated that the induction of IL-1β is mediated through ROS generation (Fig. 3d).

LPA induces IL-1β expression in human macrophages

To examine if the effects of LPA are consistent in the human system, human macrophages derived from umbilical cord blood were used. At the mRNA level, IL-1β was elevated as early as 2 h, and the expression level was sustained by 5 μM LPA at 4 h (Fig. 4a). Similar results were also observed in PMA-activated THP-1 cells (data not shown). On the other hand, the IL-1β protein was substantially enhanced from 1.21 to 2.58 pg/ml when cells were incubated with 5 μM LPA for 8 h (Fig. 4b).

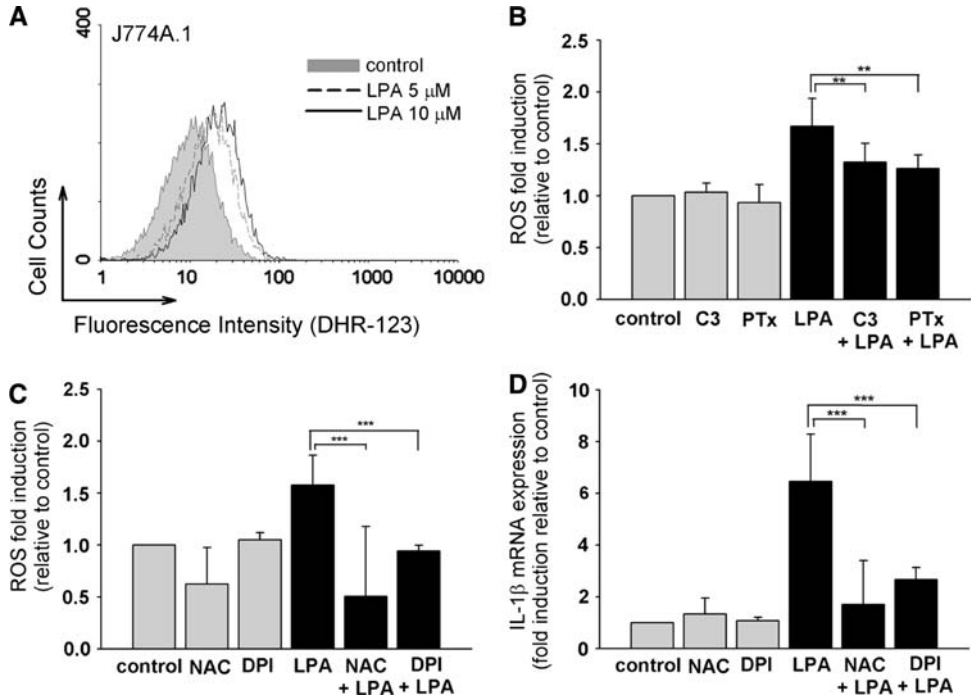

Fig. 3 Lysophosphatidic acid (LPA)-induced interleukin (IL)-1β is mediated through reactive oxygen species (ROS) production. To determine intracellular ROS production, starved J774A.1 cells were treated as described in "Materials and methods". (**a**) Histograms from the flow cytometric analysis represent the fluorescence intensity of DHR-123 in cells treated with phosphate-buffered saline (PBS) (gray shading), 5 μM LPA (dashed line), or 10 μM LPA (black line). (**b**) J774A.1 cells were pretreated with 1 μg/ml C3 or 15 ng/ml pertussis toxin (PTx) overnight followed by 10 μM LPA treatment for 30 min. (**c, d**) J774A.1 cells were pretreated with 10 mM NAC or 10 μM DPI for 1 h. For ROS measurement, pretreated cells were incubated with 10 μM LPA for 30 min and subjected to flow cytometric analysis (**c**). To evaluate IL-1β mRNA levels, pretreated cells were treated with 1 μM LPA for 2 h and mRNAs were analyzed by real-time PCR (**d**). $^{**}P < 0.01$, $^{***}P < 0.001$

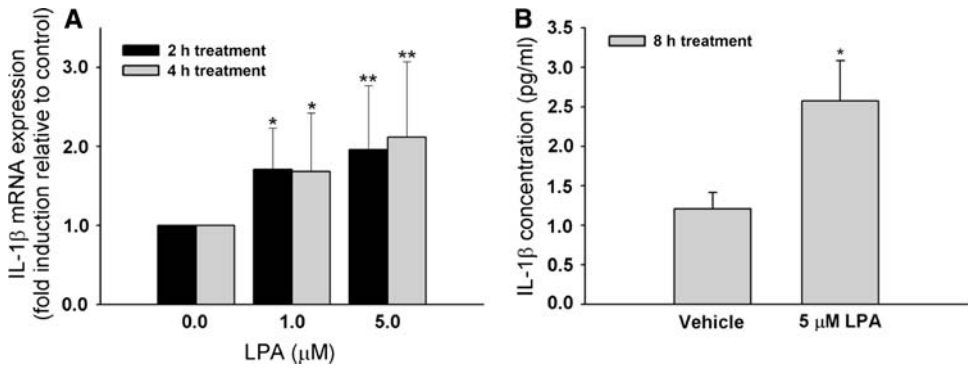

Fig. 4 Lysophosphatidic acid (LPA) induces interleukin (IL)-1β expression in human macrophages. Starved human macrophages derived from umbilical cord blood were treated as indicated. (**a**) mRNA from treated cells was harvested and subjected to real-time RT-PCR analysis. (**b**) Conditioned media were subjected to an ELISA analysis. $^{*}P < 0.05$, $^{**}P < 0.01$

Discussion

Only limited reports have been published describing the actions of LPA on the inflammatory functions of macrophages. Previous studies showed that LPA induces calcium mobilization and ROS production in human monocytic cells [4, 10], as well as acting as a survival factor in mouse macrophages [17]. Moreover, ROS production stimulated by LPA in other cell types has also been reported [3, 29]. On the other hand, it is suggested that G_i or G_{12}-Rho activation is also involved in ROS production [11, 15, 25], and the increased level of ROS could effectively enhance IL-1β expression in J774A.1 cells when treated with LPS [14]. Our current study provides evidence for the first time that LPA induces IL-1β expression and ROS production in both mouse and human macrophages. We also showed that LPA-induced IL-1β is affected by G_i, Rho, and ROS inhibitors. Compared with the molecular mechanisms exerted by other stimuli, such as LPS, ligands of scavenger receptors, and thrombin [13, 14, 24], LPA might act as a novel and critical modulator of IL-1β production in macrophages. All effects elicited by LPA discussed in this

report could be substantially observed with 1–5 μM LPA treatments, which are considered physiological concentrations in healthy human plasma [33]. Furthermore, local concentration of LPA could be significantly elevated in wound site and atherosclerotic lesion as a result of platelet activation and oxidized low-density lipoprotein (LDL) accumulation, respectively [30]. Therefore, the activation of macrophages by LPA present in this study might be physiologically relevant.

The proinflammatory roles of IL-1β are well characterized [6]. However, it was suggested that there is a dissociation between the transcription and translation of IL-1β despite mRNA being substantially synthesized [7]. Our results showed that LPA upregulates IL-1β expression at both the transcriptional and translational levels, and also showed a novel observation of IL-1β induction in human primary macrophages. The timing of IL-1β expression was also in agreement with previous reports [7, 14], which indicates the direct effects of LPA rather than indirect actions of other mediators regulated by LPA. These results suggest that LPA-induced IL-1β not only acts as an autocrine mediator, but also activates surrounding endothelial cells at the wound site or in atherosclerotic lesions.

Several reports have suggested that macrophages derived from different species might respond inconsistently [23, 27, 28]. However, our data indicated similarities of LPA effects in macrophages originating from different species, and therefore provide crucial information that mouse macrophages might be utilized as a model in interpreting the functions of LPA. Lipid-laden macrophages, also known as foam cells, play crucial roles in the process of atherosclerosis, an inflammation-related disease [26]. Increased foam cell lesions in IL-1ra knockout mice have been reported [5], which indicates that the IL-1 cytokine system is essential to atherogenesis. Moreover, in human endothelial cells, IL-1β is involved in LPA-induced monocyte chemoattractant expressions of IL-8 and MCP-1, which leads to a self-augmented inflammation process [21]. However, the relationship of LPA-induced IL-1β and other cytokines/chemokines in macrophages is currently unknown. In addition to IL-1β, it was suggested that ROS are also important regulators of inflammation [9, 16], and to some extent, facilitate atherogenesis by oxidation of LDL [12]. Oxidized LDL is the main source of LPA in atherosclerotic lesions [30], which might further deteriorate atherogenesis by activating macrophages. Therefore, these results provide essential information on the effects of LPA in macrophages.

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